

## **Proteomic Analysis of Apoptotic Pathways Reveals Prognostic Factors in Follicular Lymphoma**

Christian Gulmann,<sup>1</sup> Virginia Espina,<sup>1</sup> Emanuel Petricoin III,<sup>3</sup> Dan L. Longo,<sup>4</sup> Mariarita Santi,<sup>5</sup> Turid Knutsen,<sup>2</sup> Mark Raffeld,<sup>1</sup> Elaine S. Jaffe,<sup>1</sup> Lance A. Liotta,<sup>1</sup> and Andrew L. Feldman<sup>1</sup>

**Abstract** Follicular lymphoma (FL) is the second most common non-Hodgkin's lymphoma and generally is incurable. Reliable prognostic markers to differentiate patients who progress rapidly from those who survive for years with indolent disease have not been established. Most cases overexpress Bcl-2, but the pathogenesis of FL remains incompletely understood. To determine whether a proteomic approach could help overcome these obstacles, we procured lymphoid follicles from 20 cases of FL and 15 cases of benign follicular hyperplasia (FH) using laser capture microdissection. Lysates were spotted on reverse-phase protein microarrays and probed with 21 antibodies to proteins in the intrinsic apoptotic pathway, including those specific for posttranslational modifications such as phosphorylation. A panel of three antibodies [phospho-Akt(Ser473), Bcl-2, and cleaved poly(ADP-ribose) polymerase] segregated most cases of FL from FH. Phospho-Akt(Ser473) and Bcl-2 were significantly increased in FL ( $P = 0.001$  and  $P < 0.0001$ , respectively). Additionally, the Bcl-2/Bak ratio completely segregated FL from FH. High ratios of Bcl-2/Bak and Bcl-2/Bax were associated with early death from disease with differences in median survival times of 7.3 years ( $P = 0.0085$ ) and 3.8 years ( $P = 0.018$ ), respectively. Using protein microarrays, we identified candidate proteins that may signify clinically relevant molecular events in FL. This approach showed significant changes at the posttranslational level, including Akt phosphorylation, and suggested new prognostic markers, including the Bcl-2/Bak and Bcl-2/Bax ratios. Proteomic end points should be incorporated in larger, multicenter trials to validate the clinical utility of these protein microarray findings.

Follicular lymphoma (FL) is a common and essentially incurable B-cell malignancy that exhibits marked clinical heterogeneity (1). Some patients undergo rapid transformation to aggressive lymphoma and die, whereas others survive for years with indolent disease. Even in this latter group, the clinical course usually is characterized by multiple relapses and ultimately progression and death (2). Reliable prognostic markers have not been established to guide therapy for patients with FL. Pathologic grading lacks adequate reproducibility (3), and the clinically based International Prognostic Index identifies relatively few high-risk patients (4). A recent modification of the International Prognostic Index for use in

FL has been proposed (5) but its clinical utility has not yet been shown.

No treatment is accepted as conferring a clinically meaningful survival advantage to FL patients (1). Targeted therapies offer new hope by mitigating the effects of molecular defects in the malignant cells; these cells harbor the t(14;18) translocation in most patients with FL, leading to overexpression of Bcl-2 oncoprotein and inhibition of apoptosis (6). Although Bcl-2 is an attractive target for therapy (7), Bcl-2 overexpression alone does not fully explain the pathogenesis of FL. Of FL cases, 10% to 20% lack Bcl-2 overexpression and yet still exhibit inhibition of apoptosis (8, 9). Furthermore, mice overexpressing Bcl-2 under the control of an immunoglobulin H enhancer develop follicular hyperplasia (FH) but not FL (10). Rare t(14;18)<sup>+</sup> cells can be detected in nonmalignant human tissues (11), and we and others have reported focal colonization of lymphoid follicles by t(14;18)<sup>+</sup>/Bcl-2<sup>+</sup> cells without evidence of systemic FL (12, 13). These data suggest that pathogenetic mechanisms other than Bcl-2 overexpression exist in FL. Molecular events including p53 alterations accompany transformation of FL to aggressive lymphoma (14), but earlier events that determine which patients will progress rapidly are unknown.

Recent gene expression profiling studies have identified multigene signatures that correlate with FL outcome (15, 16). Gene expression profiling, however, may not be able to assess the activation status of apoptotic pathways, which depend on posttranslational events including phosphorylation, cleavage, and proteasomal degradation of the involved proteins (17–19).

**Authors' Affiliations:** <sup>1</sup>National Cancer Institute–Food and Drug Administration Clinical Proteomics Program, Laboratory of Pathology, and <sup>2</sup>Genetics Branch, National Cancer Institute; <sup>3</sup>Tissue Proteomics Unit, Division of Therapeutic Proteins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland; <sup>4</sup>National Institute on Aging, Baltimore, Maryland; and <sup>5</sup>Department of Pathology, Children's National Medical Center, Washington, District of Columbia

Received 3/21/05; revised 5/13/05; accepted 5/31/05.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Andrew L. Feldman, Laboratory of Pathology, National Cancer Institute, Room 2A33, Building 10, 10 Center Drive Bethesda, MD 20892. Phone: 301-594-2945; Fax: 301-402-2415; E-mail: feldmana@mail.nih.gov.

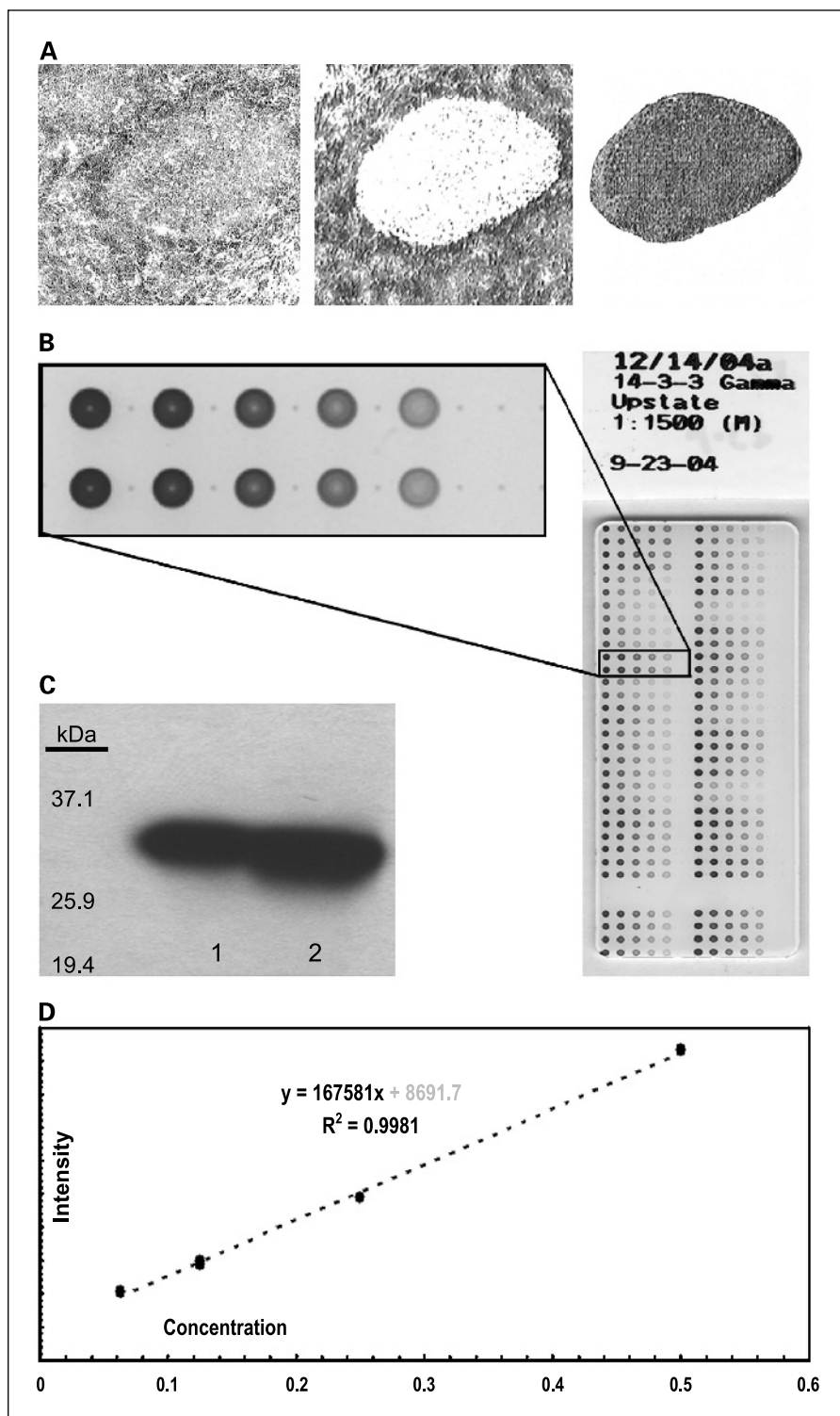
© 2005 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-0637

No adequate *in vitro* model exists for proteomic analysis of apoptotic pathways in FL. Because the median survival of FL is 8 to 10 years (2), archived frozen tissue samples with informative survival data are scarce. Therefore, we undertook a small study of material from our institution to determine whether protein microarray analysis of apoptotic pathways might be a useful tool to identify prognostic factors in FL, thus warranting a larger, multicenter study.

## Materials and Methods

**Patient samples.** We studied archived frozen tissue from 20 cases of FL (obtained between 1981 and 1989 to allow adequate follow-up time) and 15 cases of FH (5 reactive lymph nodes and 10 pediatric tonsils). Material was obtained under Institutional Review Board–approved protocols at the NIH (Bethesda, MD) or Children’s National Medical Center (Washington, DC) with informed consent. FL patients were t(14;18)<sup>+</sup> by cytogenetic studies, done as previously described



**Fig. 1.** Schema for generating microarray data. **A**, lymphoid follicles were procured using laser capture microdissection (from left to right: hematoxylin-stained lymphoid follicle, tissue remaining after removing microdissected follicle, and microdissected follicle on polymer-coated cap). **B**, protein lysates from microdissected follicles were printed using a robotic arrayer in duplicate dilution series (neat, 1:2, 1:4, 1:8, 1:16, lysis buffer control). Arrays then were probed with specific antibodies; in this case, for 14-3-3 $\gamma$ . **C**, each antibody was validated by Western blot. The protein shown is 14-3-3 $\gamma$  (30 kDa): lane 1, A431 cell lysate; lane 2, follicular lymphoma lysate. **D**, arrays were scanned and mean pixel intensity was plotted against lysate concentration. Relative protein expression was calculated using the regression equation corresponding to the linear portion of the dilution curve.

**Table 1.** Antibodies used in reverse phase protein microarrays

Antibody*	Species	Source	Western blot dilution	Protein microarray dilution
14-3-3γ	Mouse	Upstate	1:10,000	1:1,500
Akt	Rabbit	Cell Signaling	1:1,000	1:250
pAkt (Thr308)	Rabbit	Cell Signaling	1:1,000	1:100
pAkt (Ser473)	Rabbit	Cell Signaling	1:1,000	1:200
Aurora A	Rabbit	Cell Signaling	1:1,000	1:250
Bad	Rabbit	Cell Signaling	1:500	1:100
Bak	Rabbit	Cell Signaling	1:1,000	1:150
Bax	Rabbit	Cell Signaling	1:1,000	1:250
Bcl-2	Mouse	DAKO	1:2,000	1:1,000
pBcl-2 (Thr56)	Rabbit	Cell Signaling	1:1,000	1:100
pBcl-2 (Ser70)	Rabbit	Cell Signaling	1:1,000	1:250
Bcl-xL	Rabbit	Cell Signaling	1:1,000	1:500
Caspase 3	Rabbit	Cell Signaling	1:500	1:250
cCaspase 3 (Asp175)	Rabbit	Cell Signaling	1:250	1:250
Caspase 8	Mouse	Cell Signaling	1:1,000	1:200
Mcl-1	Rabbit	Cell Signaling	1:1,000	1:50
PARP	Rabbit	Cell Signaling	1:1,000	1:500
cPARP (Asp214)	Rabbit	Cell Signaling	1:1,000	1:200
Smac/DIABLO	Mouse	Cell Signaling	1:1,000	1:500
Survivin	Mouse	Cell Signaling	1:1,000	1:200
XIAP	Rabbit	Cell Signaling	1:1,000	1:1,000

Abbreviation: PARP, poly(ADP-ribose) polymerase.  
 \*p, phosphorylated at residue shown; c, cleaved at residue shown.

(20). Clinical data were obtained by medical record review. FL diagnoses were confirmed using WHO criteria (21) incorporating morphologic, immunophenotypic, and cytogenetic findings. Additional FL cases were studied using dual immunostaining procedures based on availability of formalin-fixed, paraffin-embedded tissue.

**Construction of reverse-phase protein microarrays.** Using laser capture microdissection (Pixcell II, Arcturus Bioscience, Mountain View, CA; ref. 22), ~6,000 laser shots (estimated >20,000 cells) were obtained from lymphoid follicles in each sample. Tissue processing and lysate preparation have been previously described (23, 24). Lysates were loaded into 384-well plates in serial dilutions (neat, 1:2, 1:4, 1:8, and 1:16) with negative control wells containing lysis buffer only. Dilution series of samples and a reference standard were printed in duplicate onto nitrocellulose-coated glass slides (Schleicher & Schuell Bioscience, Keene, NH) using a ring-and-pin robotic arrayer (GMS 417, Affymetrix, Santa Clara, CA). The reference standard was prepared from Fas ligand-treated Jurkat cells; human peripheral blood lymphocytes treated with pervanadate, IFN-α, granulocyte/macrophage colony-stimulating-factor, or phorbol 12-myristate 13-acetate; and human prostate cancer cryosections. This standard has shown strong staining with a wide variety of antibodies in prior array experiments. Additional arrays were printed using known concentrations of recombinant human Bcl-2 (Oncogene Research Products, Cambridge, MA) to provide further validation.

**Immunostaining and analysis of reverse-phase protein microarrays.** Microarrays were stained as previously described (23) on an autostainer (DAKO, Carpinteria, CA) using a biotinyl-linked catalyzed signal amplification system (DAKO). Antibodies were validated by Western blot as previously described (25) and concentrations were optimized using test arrays containing FL and tonsil lysates. Stained arrays were scanned on a UMEX flatbed scanner. Total protein content of each array spot was detected using Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) and a fluorescence imaging system (Alpha

Innotech, San Leandro, CA). Mean pixel intensities were calculated with background correction using ImageQuant software (v. 5.2, Molecular Dynamics, Piscataway, NJ). For each sample, the slope of the regression line best fitting the linear range of the dilution curve was used to determine relative protein expression (26). Data were normalized to total protein and to the reference standard.

**Immunohistochemical staining of tissue sections.** CD20, CD3, and Bcl-2 immunostains were done as previously described (9). T cells within neoplastic follicles were counted in 10 representative high-power fields. Double immunostaining was done on deparaffinized formalin-fixed tissue sections as previously described (27) with minor modifications. Briefly, after 8 minutes microwave antigen retrieval (High pH Target Retrieval Solution, DAKO) slides were blocked in TBS containing 3% goat serum (Invitrogen). Sections were incubated with rabbit polyclonal anti-Bak antibody (1:100; Cell Signaling, Beverly, MA) for 2 hours and with mouse monoclonal antibody against either Pax-5 (1:200; Becton Dickinson, San Jose, CA) or ZAP-70 (1:2,000; Upstate, Charlottesville, VA) for 60 minutes. Signal detection was done on an autostainer (DAKO) using species-specific EnVision<sup>+</sup>-labeled polymer-horseradish peroxidase antibody and either 3,3'-diaminobenzidine tetrahydrochloride or Vector VIP peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Images were acquired using an Olympus DP12 camera and BX50 microscope (Olympus, Melville, NY).

**Statistical methods.** Hierarchical clustering was done using JMP 5.0 (SAS Institute, Cary, NC) with the approach adopted for cDNA microarrays (28). Because most proteins were similarly expressed in FH and FL, supervised analysis was carried out using principal component analysis. The proteins that contributed most to the scatter of the data (highest absolute values) were chosen from the eigenvector that visually best separated FL from FH (29). Hierarchical clustering was repeated using panels of varying numbers of high-scoring proteins to find the panel that best differentiated the two groups. Partition analysis (30, 31) was done using JMP. Data are presented as means ± SDs,

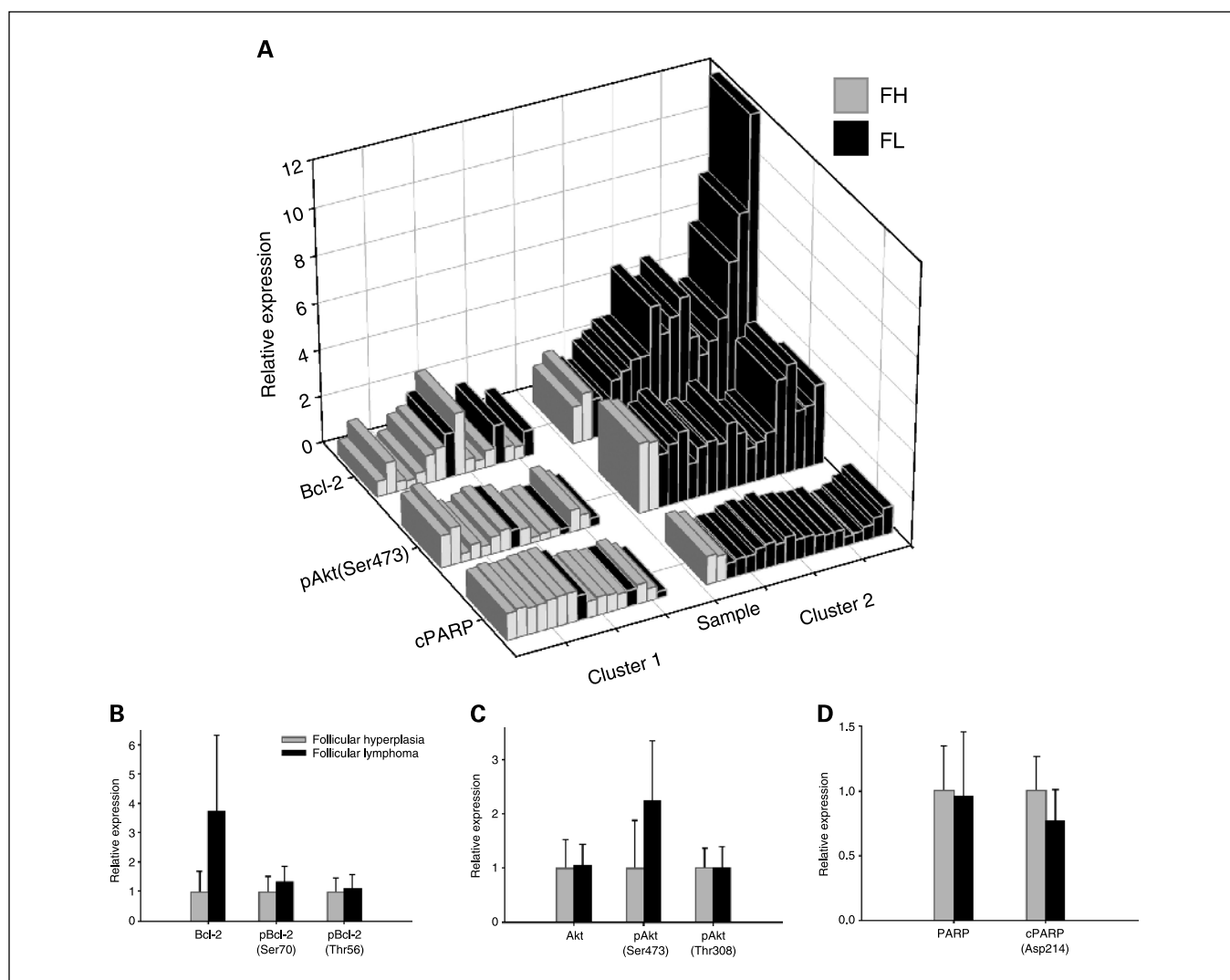
except survival data, which are presented as medians. Significance was defined as  $P < 0.05$ ; for analyses involving the panel of 21 antibodies, significance was defined as  $P \leq 0.002$  to correct for multiple comparisons. Comparisons were analyzed using the Mann-Whitney test or Spearman rank correlation. Survival data were plotted using the Kaplan-Meier method and analyzed using the log-rank (Mantel-Cox) test. Analyses were done using JMP 5.0 or StatView (SAS Institute).

## Results

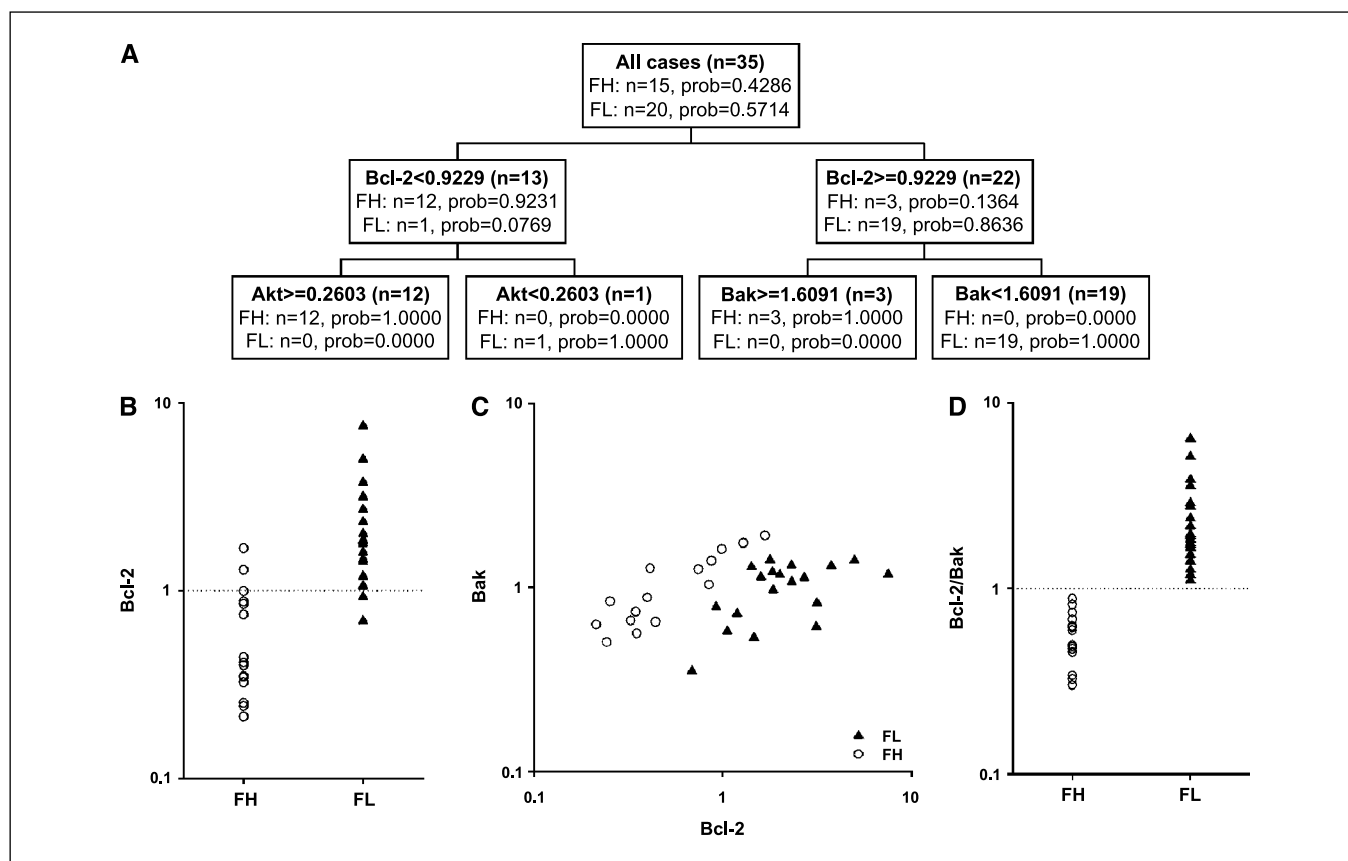
Lymphoid follicles were isolated from cases of FL and FH using laser capture microdissection (Fig. 1A). Reverse-phase protein microarrays were constructed (Fig. 1B) and probed with antibodies to 21 proteins related to apoptosis, including phosphorylated and cleared forms (Table 1). All antibodies were validated by Western blot (Fig. 1C). Arrays were scanned and dilution curves were used to quantitate relative protein expression (Fig. 1D). Test arrays showed mean intraarray and

interarray coefficients of variation of 7.8% and 7.7%, respectively. Sensitivity was  $<50 \text{ fg}/\mu\text{L}$ .

**Principal component analysis segregates follicular lymphoma from follicular hyperplasia.** Principal component analysis was done to determine the panel of antibodies that could best differentiate FL from FH. This analysis identified Bcl-2, phospho-Akt(Ser473), and cleaved poly(ADP-ribose) polymerase as the strongest discriminators. Hierarchical clustering based on this panel separated the cases into two groups, one containing 13 FH cases and 3 FL cases and the other containing 17 FL cases and 2 FH cases (Fig. 2A). As expected, total Bcl-2 was significantly overexpressed in FL (expression relative to FH,  $3.73 \pm 2.59$ ;  $P < 0.0001$ ; Fig. 2B). Phospho-Bcl-2 expression was similar to FH (Ser70,  $1.32 \pm 0.54$ ; Thr56,  $1.11 \pm 0.47$ ). Phospho-Akt(Ser473) was overexpressed in FL ( $2.25 \pm 1.11$ ;  $P = 0.001$ ) but total Akt and phospho-Akt(Thr308) were not ( $1.05 \pm 0.38$  and  $1.02 \pm 0.37$ , respectively; Fig. 2C).



**Fig. 2.** Principal component analysis of follicular lymphoma and follicular hyperplasia. **A**, based on the results of principal component analysis, hierarchical clustering was done using expression values for Bcl-2, phospho-Akt(Ser473), and cleaved poly(ADP-ribose) polymerase (cPARP). Cases were segregated into two groups, one consisting of 13 FH cases and 3 FL cases (cluster 1), and the other consisting of 17 FL cases and 2 FH cases (cluster 2). **B**, total Bcl-2, but not phosphorylated Bcl-2, was overexpressed in FL (black bars) compared with FH (gray bars;  $P < 0.0001$ ). Values are shown standardized to a mean FH value of 1.0. **C**, phospho-Akt(Ser473) was overexpressed in FL ( $P = 0.001$ ), but expression of total Akt and phospho-Akt(Thr308) was similar to FH. **D**, there was a trend toward lower cleaved poly(ADP-ribose) polymerase in FL cases ( $P = 0.023$ ).



**Fig. 3.** Partition analysis of follicular lymphoma and follicular hyperplasia. *A*, partition analysis was used to develop a model that discriminated FL from FH based on relative expression values of Bcl-2, Akt, and Bak. *B*, Bcl-2 was the major discriminator of FL versus FH; however, an area of overlap (values around 1.0) was noted. *C*, plotting Bcl-2 expression against Bak expression segregated the cases of FL and FH into discrete groups. *D*, the Bcl-2/Bak ratio completely segregated FL and FH cases, with high ratios corresponding to FL.

Phospho-Akt(Ser473) expression did not correlate significantly with Bcl-2 expression ( $R = 0.35$ ;  $P = 0.13$ ). Cleaved poly(ADP-ribose) polymerase was decreased in FL ( $0.77 \pm 0.24$ ; Fig. 2D) although this was not significant when corrected for multiple comparisons ( $P = 0.023$ ). Total poly(ADP-ribose) polymerase expression was similar in FL and FH.

**Partition analysis segregates follicular lymphoma from follicular hyperplasia.** Partition analysis was used to develop a model that completely separated FL and FH on the basis of Bcl-2, Akt, and Bak expression values (Fig. 3A). Although Bcl-2 expression was the dominant component of this model, Bcl-2 expression alone showed a region of overlap between FL and FH (Fig. 3B). Bak played a greater contributing role than Akt, and a two-dimensional plot of Bcl-2 versus Bak showed discrete clustering of FL and FH cases (Fig. 3C). The Bcl-2/Bak ratio completely segregated FL from FH, with increased Bcl-2/Bak ratios corresponding to FL (Fig. 3D).

**Increased Bcl-2/Bax and Bcl-2/Bak ratios are associated with early death from follicular lymphoma.** Clinicopathologic data from the FL patients are shown in Table 2. Follow-up ranged from 1.1 to 15.2 years from time of biopsy. Median overall survival was 9.3 years. Neither stage at diagnosis nor administration of chemotherapy before biopsy was significantly associated with survival. There were trends toward shorter survival times among patients with a histologic grade of 3 or a diffuse component, but these differences were not statistically

significant. No individual protein showed a statistically significant association with survival.

Because the Bcl-2/Bax ratio has been reported to regulate apoptosis (32) and correlate with outcome in other tumors (33), and Bcl-2/Bak ratios segregated FL from FH in the present study, we investigated the potential prognostic significance of these ratios in FL. Using the median Bcl-2/Bax ratio of 2.90 as a cutoff value, increased Bcl-2/Bax ratios were associated with significantly shorter survival times (median, 7.6 versus 11.4 years;  $P = 0.018$ ; Fig. 4A). The mean Bcl-2/Bax ratio was  $1.00 \pm 0.40$  in FH,  $1.97 \pm 0.44$  in FL with good outcome (survival  $\geq 10$  years), and  $5.33 \pm 4.30$  in FL with poor outcome (survival  $< 10$  years, excluding censored cases; see Fig. 4B for  $P$  values). Using the median Bcl-2/Bak ratio of 1.87 as a cutoff value, increased Bcl-2/Bak ratios were associated with significantly shorter survival times (median, 4.1 versus 11.4 years;  $P = 0.0085$ ; Fig. 4C). Bcl-2/Bak ratios were  $0.59 \pm 0.17$  in FH,  $1.55 \pm 0.27$  in FL with good outcome, and  $2.94 \pm 1.73$  in FL with poor outcome (see Fig. 4D for  $P$  values). Bcl-2, Bax, and Bak were not significantly associated with outcome when analyzed individually.

**Bak is expressed in the neoplastic cells of follicular lymphoma.** The neoplastic B cells of FL express Bcl-2 and Bax (9). The expression pattern of Bak in FL has not been reported. The neoplastic follicles of FL contain both malignant B lymphocytes and nonneoplastic immune cells (21). To determine which

cells express Bak in the neoplastic follicles of FL, we did dual immunostains for Bak and either ZAP-70 (expressed in the nuclei of reactive T cells; ref. 34) or Pax-5 (a B-cell transcription factor expressed in the neoplastic cells of follicular lymphoma; ref. 27). Bak was expressed both in neoplastic B cells of FL (Fig. 4E) and in nonneoplastic T cells (Fig. 4F). The microarray values for Bak expression did not correlate significantly with the T-cell density in the neoplastic follicles ( $R = -0.27$ ;  $P = 0.24$ ).

## Discussion

This study shows the potential of protein microarrays to identify prognostic markers in FL, a B-cell lymphoma characterized by inappropriate inhibition of apoptosis (9, 35). Apoptotic pathways represent one important determinant of cell fate, and the activation of these pathways depends on posttranslational events that may not be reflected in gene expression profiles. Therefore, we employed a proteomic approach to study FL, using reverse-phase protein microarrays (25) to allow comparison of protein expression in multiple tissue samples simultaneously. This technique shows high sensitivity due to signal amplification via horseradish peroxidase-mediated biotinyl-tyramide deposition. Printing serial dilution curves of each sample allows analysis in the linear range of each curve, and enhances the ability to analyze the broad range of protein concentrations present in the human proteome (compared with, for example, RNA concentrations; refs. 36, 37).

Hierarchical clustering of cDNA microarray data has played a pivotal role in elucidating lymphoma biology. In addition to identifying prognostic gene expression profiles in FL (15, 16),

this approach has been used to characterize the cytokine milieu of the FL microenvironment (38), identify profiles that differentiate FL from other B-cell lymphomas (39, 40), and characterize gene expression profiles associated with the t(14;18) translocation (41). Similarly, clustering has greatly facilitated identification of biologically relevant differences in protein expression using reverse-phase protein microarrays (42). Future coupling of gene expression and proteomic profiling may provide an even more comprehensive and powerful approach to molecular profiling of lymphoma.

In this study, we used hierarchical clustering to evaluate antibody panels identified by principal component analysis. FL recapitulates the biology and morphology of reactive lymphoid follicles, and most proteins examined in this study were similarly expressed in FL and FH. Nevertheless, a panel of three antibodies segregated most cases of FL from FH. Not surprisingly, this panel included the antibody to Bcl-2; interestingly, however, expression of phospho-Bcl-2 was similar in FL and FH. Some studies indicate that phospho-Bcl-2 loses its antiapoptotic function and is targeted for proteasomal degradation (43, 44). Despite increased expression of total Bcl-2 in FL, the phosphorylated fraction does not seem to be proportionately increased.

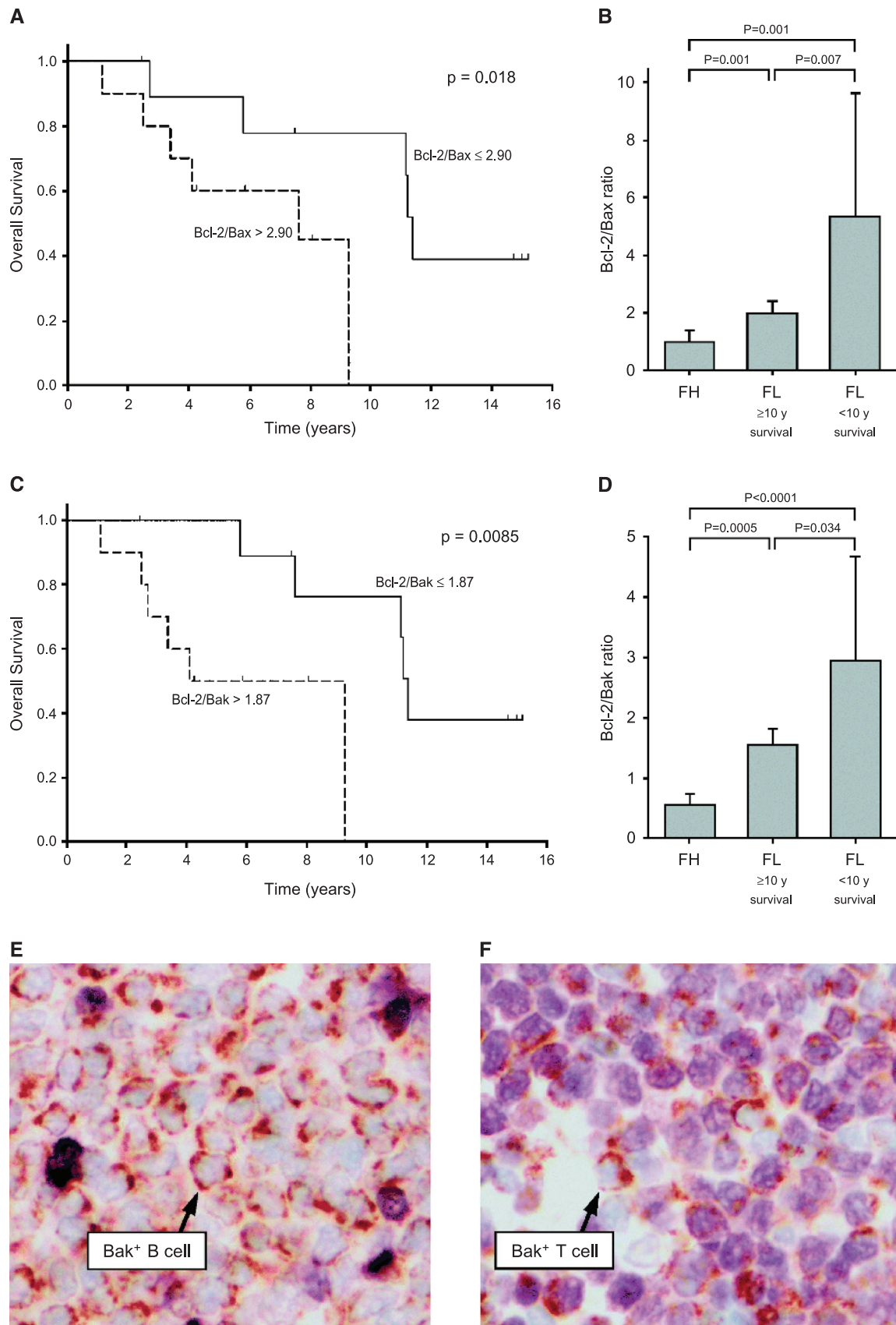
Phospho-Akt(Ser473) was increased in FL, whereas total Akt and phospho-Akt(Thr308) did not differ from FH. These findings support data from a smaller, separate study which showed increased phospho-Akt(Ser473) expression in FL but did not examine phospho-Akt(Thr308) (9). Akt inhibits apoptosis and is activated by phosphorylation via the phosphatidylinositol 3'-OH kinase cascade (45). An association between Akt activity and selective phosphorylation at

**Table 2.** Clinicopathologic data for 20 patients with follicular lymphoma

Case	Age*	Sex	Clinical stage	Prior chemotherapy	Histologic grade	Diffuse component	Follow-up (y)	Disease status <sup>†</sup>
1	35	M	4	Yes	3	No	4.1	DOD
2	23	M	3	No	2	No	15.2	NED
3	32	F	4	No	1	No	15.0	NED
4	59	F	4	Yes	3	No	2.5	AWD
5	31	M	4	No	1	No	11.2	DOD
6	55	M	4	Yes	3	Yes	5.9	NED
7	30	F	4	Yes	2	No	7.6	DOD
8	46	M	3	No	2	Yes	5.8	DOD
9	67	M	3	Yes	3	Yes	1.1	DOD
10	64	M	3	Yes	2	No	14.7	NED
11	35	M	3	Yes	1	No	3.4	DOD
12	39	M	4	No	2	No	9.3	DOD
13	34	F	4	No	1	No	8.1	AWD
14	56	F	4	Yes	2	No	11.2	DOD
15	25	F	4	Yes	1	No	11.4	DOD
16	40	F	4	Yes	2	No	2.5	DOD
17	56	M	3	No	2	No	2.7	DFD
18	51	F	3	Yes	1	No	4.3	AWD
19	65	F	3	No	2	No	9.3	DOD
20	46	M	4	No	2	Yes	7.5	AWD

\*Age at time of biopsy.

†DOD, died of disease; DFD, died free of disease; AWD, alive with disease; NED, no evidence of disease.





**Fig. 4.** Prognostic significance of Bcl-2/Bax and Bcl-2/Bak ratios in FL. *A*, FL patients with Bcl-2/Bax ratios above the median value died sooner than those with lower ratios ( $P = 0.018$ ). Neither Bcl-2 nor Bax alone had significant prognostic value. *B*, Bcl-2/Bax ratios were highest in FL patients with poor prognosis (death within 10 years). *P* values are shown. *C*, the Bcl-2/Bak ratio was an even stronger prognostic marker. FL patients with high ratios died sooner than those with low ratios, with a 7.3-year difference in median overall survival ( $P = 0.0085$ ). *D*, Bcl-2/Bak ratios also were highest in FL patients with poor prognosis. *E*, Bak protein was shown immunohistochemically as granular brown cytoplasmic staining in the neoplastic B cells of FL (arrow). The scattered cells with purple nuclear staining are nonneoplastic T cells stained for ZAP-70. *F*, nonneoplastic T cells also express Bak protein (arrow). The numerous surrounding cells with purple nuclear staining are the neoplastic B cells of FL stained for Pax-5.

Ser473, but not at Thr308, has been reported in colorectal cancer (46). In our previous study, phospho-Akt expression was increased in Bcl-2-negative FL as well as Bcl-2-positive FL (9). In the present study, phospho-Akt(Ser473) expression did not correlate significantly with Bcl-2 expression. Taken together, these findings suggest that Akt activation may be an important signaling event in FL that functions independently of Bcl-2.

This is the first study to suggest the biological importance of the Bcl-2/Bak ratio in FL. Bcl-2/Bak ratios completely discriminated FL from FH, and patients with high ratios died significantly sooner than patients with low ratios. Bak is a conserved homologue in the Bcl-2 protein family that acts at the mitochondrial membrane to facilitate release of cytochrome *c*, triggering caspase activation and apoptosis (47). Bcl-2 inhibits the proapoptotic effects of Bak by preventing Bak oligomerization (48). Bak expression has been reported to be low in FL (49), and the cell types producing Bak in FL have not been previously reported. We showed that Bak is expressed in the neoplastic B cells of FL as well as in intermixed reactive T cells. Because T-cell density did not correlate with overall Bak expression, the prognostic significance of the Bcl-2/Bak ratio likely relates to coexpression of Bcl-2 and Bak in malignant B cells.

Bax is another proapoptotic Bcl-2 family member related structurally and functionally to Bak (47). The Bcl-2/Bax ratio has been called a "rheostat" regulating apoptosis at the cellular level (32). Elevated Bcl-2/Bax ratios measured using flow cytometry have been associated with poor prognosis in acute myeloid leukemia (33); however, this approach is not applicable to archived frozen tissue. Reverse-phase protein

microarrays, by using lysates derived from the cell population of interest and determining relative expression from the linear portion of the dilution curve, are well suited to accurately quantitate protein ratios from archived tissue.

In summary, this study shows the potential of protein microarrays to identify clinically relevant molecular events in patients with FL. These events include posttranslational modifications, such as activation of Akt by phosphorylation, which are critical in apoptotic regulation. These initial results also suggest that Bcl-2/Bak and Bcl-2/Bax ratios may be important prognostic indicators in patients with FL. Because proteins are the direct functional effectors of apoptotic decision-making in the cell, proteins that have biological importance in FL also merit investigation as potential therapeutic targets. Recent strategies include inhibiting Bcl-2 function or expression, enhancing activity of Bak and Bax, and disrupting Akt signaling (7, 50). However, agents with proven ability to achieve these molecular alterations are not yet available in the clinic. Thus, clinical development of targeted therapeutics should be coupled with investigation of proteomic end points in larger, multicenter trials to optimize the strategy of using molecular profiles to guide clinical therapies.

## Acknowledgments

We thank Julie Wulfkühle and Valerie Calvert for advice on protein microarrays; Yifan Zhang and Shannon McCurdy for technical assistance; Katherine Sheehan and Paul Herrmann for help with data analysis; Seth Steinberg and David Levens for helpful comments on the manuscript; and Tom Fountaine and Stefania Pittaluga for help with immunohistochemical assays.

## References

- Hornig SJ. Follicular lymphoma: have we made any progress? *Ann Oncol* 2000;11:23–7.
- Ha CS, Kong JS, McLaughlin P, et al. Stage III follicular lymphoma: long-term follow-up and patterns of failure. *Int J Radiat Oncol Biol Phys* 2003;57:748–54.
- The Non-Hodgkin's Lymphoma Classification Project: a clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. *Blood* 1997;89:3909–18.
- Decaudin D, Lepage E, Brousse N, et al. Low-grade stage III-IV follicular lymphoma: multivariate analysis of prognostic factors in 484 patients—a study of the groupe d'Etude des lymphomes de l'Adulte. *J Clin Oncol* 1999;17:2499–505.
- Solal-Celigny P, Roy P, Colombat P, et al. Follicular lymphoma international prognostic index. *Blood* 2004;104:1258–65.
- Tsujimoto T, Cossman J, Jaffe ES, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 1985;228:1440–3.
- Juin P, Geneste O, Raimbaud E, Hickman JA. Shooting at survivors: Bcl-2 family members as drug targets for cancer. *Biochim Biophys Acta* 2004;1644:251–60.
- Lai R, Arber DA, Chang KL, Wilson CS, Weiss LM. Frequency of bcl-2 expression in non-Hodgkin's lymphoma: a study of 778 cases with comparison of marginal zone lymphoma and monocytoid B-cell hyperplasia. *Mod Pathol* 1998;11:864–9.
- Zha H, Raffeld M, Charboneau L, et al. Similarities of prosurvival signals in Bcl-2-positive and Bcl-2-negative follicular lymphomas identified by reverse phase protein microarray. *Lab Invest* 2004;84:235–44.
- McDonnell T, Deane N, Platt F, et al. Bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 1989;57:79–88.
- Limpens J, de Jong D, van Krieken J, et al. Bcl-2 in benign lymphoid tissue with follicular hyperplasia. *Oncogene* 1991;6:2271–6.
- Cong P, Raffeld M, Teruya-Feldstein J, Sorbara L, Pittaluga S, Jaffe ES. *In situ* localization of follicular lymphoma: description and analysis by laser capture microdissection. *Blood* 2002;99:3376–82.
- Bende RJ, Smit LA, Bossenbroek JG, et al. Primary follicular lymphoma of the small intestine:  $\alpha\beta\gamma$  expression and immunoglobulin configuration suggest an origin from local antigen-experienced B cells. *Am J Pathol* 2003;162:105–13.
- Sander CA, Yano T, Clark HM, et al. p53 mutation is associated with progression in follicular lymphomas. *Blood* 1993;82:1994–2004.
- Glas AM, Kersten MJ, Delahaye LJ, et al. Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. *Blood* 2005;105:301–7.
- Dave SS, Wright G, Tan B, et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med* 2004;351:2159–69.
- Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 2004;384:201–32.
- Datta SR, Ranger AM, Lin MZ, et al. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell* 2002;3:631–43.
- Yang Y, Yu X. Regulation of apoptosis: the ubiquitous way. *FASEB J* 2003;17:790–9.
- Whang-Peng J, Knutsen T, Jaffe ES, et al. Sequential analysis of 43 patients with non-Hodgkin's lymphoma: clinical correlations with cytogenetic, histologic, immunophenotyping, and molecular studies. *Blood* 1995;85:203–16.
- Jaffe ES, Harris NL, Stein H, Vardiman J. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2001.
- Emmert-Buck MR, Bonner RF, Smith PD, et al. Laser capture microdissection. *Science* 1996;274:998–1001.
- Wulfkühle JD, Aquino JA, Calvert VS, et al. Signal pathway profiling of ovarian cancer from human tissue specimens using reverse-phase protein microarrays. *Proteomics* 2003;3:2085–90.

24. Grubb RL, Calvert VS, Wulkhleh JD, et al. Signal pathway profiling of prostate cancer using reverse phase protein arrays. *Proteomics* 2003;3:2142–6.
25. Paweletz CP, Charboneau L, Bichsel VE, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* 2001;20:1981–9.
26. Herrmann PC, Gillespie JW, Charboneau L, et al. Mitochondrial proteome: altered cytochrome *c* oxidase subunit levels in prostate cancer. *Proteomics* 2003;3:1801–10.
27. Krenacs L, Himmelmann AW, Quintanilla-Martinez L, et al. Transcription factor B-cell-specific activator protein (BSAP) is differentially expressed in B cells and in subsets of B-cell lymphomas. *Blood* 1998;92:1308–16.
28. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–8.
29. Jolliffe T. Principal component analysis. Berlin: Springer; 1986.
30. Ciampi A, Lawless JF, McKinney SM, Singhal K. Regression and recursive partition strategies in the analysis of medical survival data. *J Clin Epidemiol* 1988;41:737–48.
31. Lu KH, Patterson AP, Wang L, et al. Selection of potential markers for epithelial ovarian cancer with gene expression arrays and recursive descent partition analysis. *Clin Cancer Res* 2004;10:3291–300.
32. Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE, Oltvai ZN. Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Semin Cancer Biol* 1993;4:327–32.
33. Del Poeta G, Venditti A, Del Principe MI, et al. Amount of spontaneous apoptosis detected by Bax/Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). *Blood* 2003;101:2125–31.
34. Wiestner A, Rosenwald A, Barry TS, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 2003;101:4944–51.
35. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1988;335:440–2.
36. Liotta LA, Espina V, Mehta AI, et al. Protein microarrays: Meeting analytical challenges for clinical applications. *Cancer Cell* 2003;3:317–25.
37. Chan SM, Ermann J, Su L, Fathman CG, Utz PJ. Protein microarrays for multiplex analysis of signal transduction pathways. *Nat Med* 2004;10:1390–6.
38. Fujii A, Oshima K, Hamasaki M, et al. Differential expression of cytokines, chemokines and their receptors in follicular lymphoma and reactive follicular hyperplasia: assessment by complementary DNA microarray. *Oncol Rep* 2005;13:819–24.
39. Maesako Y, Uchiyama T, Ohno H. Comparison of gene expression profiles of lymphoma cell lines from transformed follicular lymphoma, Burkitt's lymphoma and *de novo* diffuse large B-cell lymphoma. *Cancer Sci* 2003;94:774–81.
40. Wang J, Delabie J, Aasheim H, Smeland E, Myklebost O. Clustering of the SOM easily reveals distinct gene expression patterns: results of a reanalysis of lymphoma study. *BMC Bioinformatics* 2002;3:36.
41. Robetorye RS, Bohling SD, Morgan JW, Fillmore GC, Lim MS, Elenitoba-Johnson KS. Microarray analysis of B-cell lymphoma cell lines with the t(14;18). *J Mol Diagn* 2002;4:123–36.
42. Nishizuka S, Charboneau L, Young L, et al. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. *Proc Natl Acad Sci U S A* 2003;100:14229–34.
43. Basu A, Haldar S. Signal-induced site specific phosphorylation targets Bcl2 to the proteasome pathway. *Int J Oncol* 2002;21:597–601.
44. Haldar S, Jena N, Croce CM. Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci U S A* 1995;92:4507–11.
45. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999;13:2905–27.
46. Khor TO, Gul YA, Ithnin H, Seow HF. Positive correlation between overexpression of phospho-BAD with phosphorylated Akt at serine 473 but not threonine 308 in colorectal carcinoma. *Cancer Lett* 2004;210:139–50.
47. Kirkin V, Joos S, Zornig M. The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Acta* 2004;1644:229–49.
48. Ruffolo SC, Shore GC. BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. *J Biol Chem* 2003;278:25039–45.
49. Agarwal B, Naresh KN. Bcl-2 family of proteins in indolent B-cell non-Hodgkin's lymphoma: study of 116 cases. *Am J Hematol* 2002;70:278–82.
50. Wendel HG, De Stanchina E, Fridman JS, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* 2004;428:332–7.